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Development of PCR-based Diagnostics for Soil Borne Plant Pathogens

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The development of accurate methods for the identification and quantification of plant pathogens can be an important step in the control and eradication of diseases in field crops and other economically important plants. Although many modern methods in molecular genetics appear to offer a great deal of promise in this respect, the adaptation of such methods for use in agriculture has been remarkably slow. For example, the polymerase chain reaction (PCR) has been exploited in basic research for more than a decade, and even a Nobel prize has been awarded for its discovery. Despite this, its use as a diagnostic tool in agriculture, particularly crop production, is only beginning to be exploited (e.g., Marshall, 1996). The reasons for this range from a natural reluctance to abandon traditional methods and lack of technical expertise, to the unique problems associated with agricultural samples and ease of contamination. In addition, unlike medical diagnostics, the potential for cost recovery is much lower and, therefore, these developments have been much less attractive from a commercial perspective.

Despite these limitations, in the last few years much more attention has begun to be focussed on the development of PCR based-diagnostics for use with plant disease. Governed in large measure by the availability of research funding, our own studies have focussed on a direct application of PCR-based diagnostics in potato, initially with emphasis on *Verticillium* but most recently on related soil nematodes and their role in PED (potato early dying syndrome). In general, even though *Verticillium* can have the same symptoms as excess chemical top-killer, draught stress or mature plant senescence, the main method of detection has been field inspection. Furthermore, the common laboratory methods, although somewhat more accurate, require pathogen isolation, a process which is both time consuming (up to seven weeks) and labour intensive. The results, at best, are semiquantitative and most tests fail to adequately distinguish the different species which are present.

In view of the limitations in current testing methods and the economic significance of *Verticillium* in many crops including alfalfa, ginseng, potato and tomato, we have chosen this fungal pathogen as a model to see if we could develop effective PCR-based diagnostics which could be readily substituted for the traditional methods. The approaches which we have developed and reported on (see Robb and Nazar, 1996) clearly are faster, more accurate, less expensive and much more easily applied on a wide scale. In more recent efforts to develop effective diagnostics for nematodes we have attempted to generalize the principle approaches which we used with *Verticillium*. These approaches are summarized here as models for general application with other plant pathogens.

RIBOSOMAL RNA GENES AS TARGETS SEQUENCES FOR PCR-BASED DIAGNOSTICS

Diagnostics based on PCR amplification require the use of a target DNA sequence that is unique to the organism being assayed, resulting in a signal which is distinct for that organism. Several decades of study on gene sequences encoding the ribosomal RNAs (rRNA) of many organisms have led us and many others to the conclusion that these genes have characteristics which make them ideal targets for PCR-based diagnostics. These features include a high genomic copy number and ease of isolation, characteristics which lead to strong analytical signals and a rapid assay development. A third feature which can be exploited has proven especially important when

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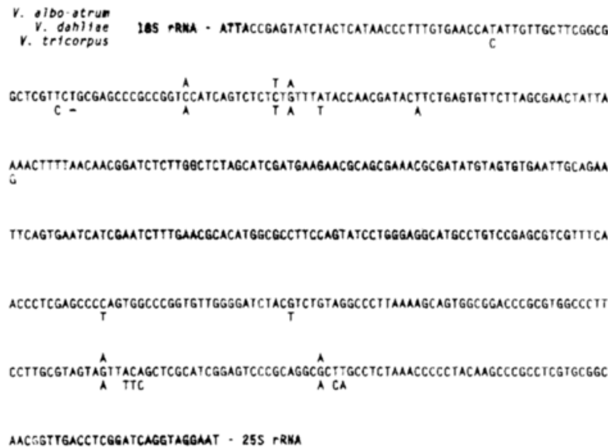

Fig. 1. Comparison of sequences for the ITS regions in genes encoding the ribosomal RNAs of *Verticillium* pathogens. The nucleotides above and below the sequence for *V. dahliae* indicate differences in *V. albo-atrum* and *V. tricorpus*, respectively. The shaded regions indicate mature rRNA sequences which are incorporated into ribosomes.

closely related species are to be differentiated. Much or all of the RNA in the ribosomes of eukaryotic organisms is transcribed initially as a much larger nucleolar precursor molecule (35-45S nRNA) which is cleaved during RNA processing to provide the mature rRNAs. As a result there are four transcribed nonconserved spacer regions in genes that encode rRNAs. As a result there are four transcribed nonconserved spacer regions in genes that encode rRNAs, two external (5' and 3' ETS) and two internal (ITS 1 and 2) spacers which separate the small (SSU) and large (LSU) subunits RNAs. Numerous phylogenetic comparisons (e.g., Nazar, 1992) have repeatedly shown that the mature rRNA sequences are highly conserved in the course of evolution, but the spacer regions which separate the mature sequences actually are very divergent, often with clear differences even between very closely related species such as *V. albo-atrum* and *V. dahliae* (e.g., Fig. 1).

To develop PCR-based diagnostics, advantage is taken of this last feature in two important ways. First, because the mature sequences are present in all organisms and are higly conserved, they can be used as targets for universal rRNA primers. As a result, useful portions of the genes can be PCR amplified from genomic DNA directly, without a need for genomic libraries and DNA cloning (e.g., Nazer *et al.*, 1991). We have used this approach extensively in the development of assays for new *Verticillium* species (e.g., Moukhamedov *et al.*, 1994) and, more recently, for nematodes. On the other hand, the presence of small sequence differences in the spacer regions of even closely related species such as *V. albo-atrum* and *V. dahliae* (Fig. 1) has enabled us not only to develop *Verticillium*-specific assays, but also to use species specific primers which allow the quantitative differentiation of these closely related *Verticillium* species (Nazer *et al.*, 1991).

Despite the large measure of success we have had in developing PCR-based diagnostics for specific *Verticillium* pathogens, out recent experiences with nematodes provide an important caution when PCR-amplification is used to evaluate new target sequences. Multicellular pathogens such as nematodes, which often cannot be isolated or grown in sterile form, can be contaminated with organisms or DNAs, either because they naturally adhere to the target or represent food. With "universal" primers DNAs from contaminating organisms also may be amplified and be mistaken for target specific DNAs. In some instances they may even be more efficiently amplified, either because there are more copies or because small sequence differences in "universal" sites result in differential amplification. Such was a problem with our nematode isolates which often gave strong signals of differing size from contaminating yeast DNAs. This fact became clear only when DNA sequence analyses were completed underlining the need for careful sample preparation and complete sequence analyses during assay developments. Size differences in PCR-amplified products from closely related organisms should, therefore, be subject to suspicion until fully confirmed by sequence analysis.

PROTOCOLS FOR DNA EXTRACTION FROM PLANT TISSUE AND SOIL SAMPLES

As noted earlier, a major limitation in application of PCR-based assays to plant pathogens has been DNA extraction. The presence of a cell wall generally makes cell disruption difficult. More important, many plant tissue extracts contain large amounts of contaminating substances which can severely inhibit or even prevent subsequent PCR amplification. This problem can be still more severe with soil samples which also can contain large amounts of inhibiting substances, or can even adsorb or degrade DNA as it is extracted. In the past, many laboratories have addressed these problems by adding additional steps of purification including many labour intensive techniques such as column chromatography or equilibrium density gradient centrifugation (see

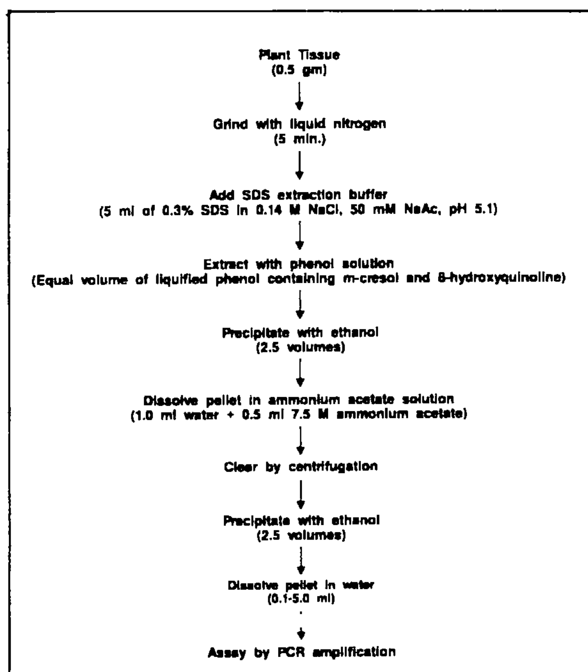

Fig. 2. Protocol for direct DNA extraction of plant tissues.

Steffan and Atlas, 1991). While such approaches often do eliminate the inhibiting effects they are costly, time consuming and frequently not quantitative. Taken together these limitations usually result in an assay which cannot be applied when large numbers of samples must be tested.

In developing PCR-based diagnostics for *Verticillium*, we adopted a very different strategy which took advantage of the very high sensitivity which is possible with PCR amplification. The resulting methods (see Figs. 2 and 3) can be applied effectively even when wide scale assays are required. Rather than remove contaminants by purification, we chose to reduce the levels of inhibitory substances by sample dilution (Nazar *et al.*, 1991; Volossiouk *et al.*, 1995) and, in the case of soil extracts (Fig. 3), we also added macromolecular carriers (Volossiouk *et al.*, 1995) to reduce DNA adsorption and degradation during DNA extraction. The only disadvantage of this simplified approach has been a reduced PCR-amplification signal, but, in most instances, the standard PCR protocol has resulted in a signal strength which is sufficient for accurate determinations. In some circumstances, particularly with soil samples when the target DNA concentration may be extremely low, we have had to increase the sensitivity be adopting a two-step or "nested" PCR protocol (Haqqui *et al.*, 1988) to detect pathogen DNA. Based on our experi-

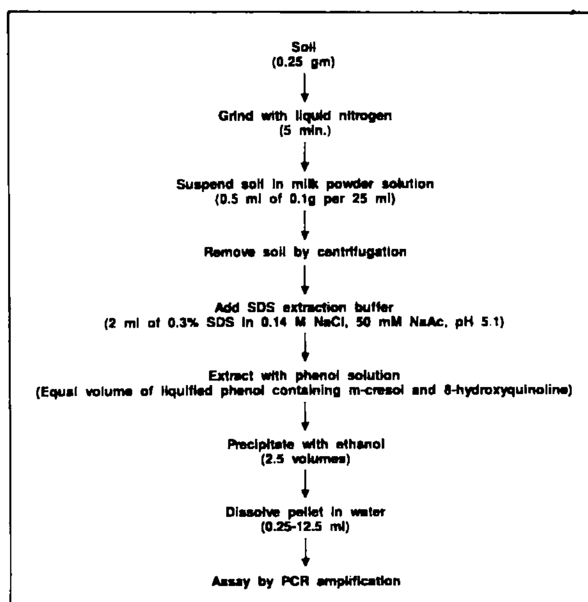

Fig. 3. Protocol for direct DNA extraction of soil samples.

ence to date, we have had to adapt the two-step protocol when a pathogen like *Verticillium,* which normally grows only in the plant, is present in soil in a resting state.

Although not ideal in every respect, the sample dilution strategy which we first used for plant tissue extracts (Fig. 2) has been adopted for both plant tissue and soil extracts, basically as a compromise which allows for low cost, rapid, wide scale applications. Normally 50-fold dilutions are entirely adequate to permit efficient PCR amplifications and the use of a basic SDS-phenol extraction protocol permits sample preparation with minimal equipment requirements (see Robb and Nazar, 1996). The basic difference for soil extracts (Fig. 3) is the addition of skim milk powder to reduce DNA loss and degradation (Volossiouk *et al.*, 1995). Both extraction protocols generally have been successful but some inhibitory effects remain in many samples, a problem which is especially critical when quantitative results are being sought. To address this problem we have turned to the use of internal control templates in the amplification reactions to fully account for inhibitory effects and to permit accurate quantifications, often not possible with traditional assays. This approach is described next.

USE OF INTERNAL CONTROL TEMPLATES FOR QUANTITATIVE ANALYSES

Control templates which are included in the PCR

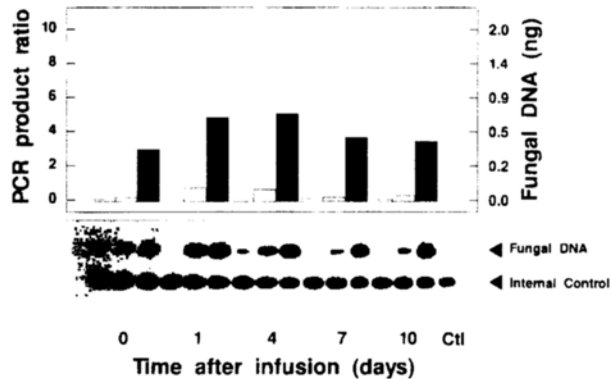

Fig. 4. Colonization of alfalfa by a non pathogenic potato isolate of *V. albo-atrum*. Three stem cuttings of a cloned plant (cv Trumpetor) were sampled at 0, 1, 4, 7 and 10 days post-inoculation. Uninoculated cuttings were used as a control (Ctl). Cuttings were divided into three portions (2.5-3.0 cm): top (open bar), middle (shaded bar) and bottom (solid bar) for DNA extraction and PCR quantification using labled nucleotides and 1 pg of internal control DNA. After fractionation by gel electrophoresis and autoradiography (lower), the amounts of fungal DNA were determined from the ratio of the fungal derived and internal control bands.

amplification mixes, result in reference signals, that serve as a quality control measure, but also can be used to accurately quantify the target-derived product. The control template contains the same primer specific sites as found in the target DNA molecule, but results in a product which is different in length and can be differentiated readily from the target-derived signal by gel electrophoresis. In the course of our studies (e.g., Hu *et al.*, 1993), we have examined internal controls which either were highly homologous or unrelated in sequence to the target pathogen (excluding the primer-specific sites). With homologous sequences we generally found many extra bands and concluded that, because genetic recombination can occur *in vitro* during PCR amplification, non-homologous sequences are more suitable. While restriction digests were used initially to prepare these internal control templates, we now prepare them routinely by PCR amplification under very non-stringent conditions (Moukhamedov *et al.*, 1994). For this purpose, an unrelated genome is randomly amplified using the target specific primers and a band of suitable length (a little shorter or larger than the target product) is selected after the products are fractionated by gel electrophoresis. This band then is normally subcloned in a common cloning vector for large scale production and plasmid, containing the internal control sequence, is prepared by equilibrium density gradient centrifugation. Because the new hybrid construct now contains the target primer sequences, it can be PCR amplified under the very stringent conditions used in a PCR-based assay.

The internal control template actually serves two purposes in the assay; it eliminates the possibility that inhibiting substances in the DNA extract give rise to false negative reactions and it also serves to calibrate the reaction efficiency, enabling quantitative determinations. For this purpose a calibration curve is constructed by using a constant concentration of internal control template in all reaction mixtures and increasing amount of target genomic DNA. The amounts of target DNA product and internal control product are determined after fractionation by gel electrophoresis (see Fig. 4) using densitometry or radioactivity and the calibration curve is prepared by plotting the ratio of target DNA product/internal template product versus the actual target genomic DNA concentration. This ratio is also calculated for all experimental samples and then each is used to determine the target concentration by comparison with the calibration curve (Hu *et al.*, 1993).

Studies to date indicate this approach is very effective when the sample dilution and internal control concentration are adjusted properly. Normally the concentration of the control template is first adjusted to give a signal which is 10-20% of the maximum that can be obtained with unlimited template. This ensures that the reaction is not saturated with control template and allows for a wide range of target DNA amplification. The intensity of the control template product is then noted in all experimental reactions. Samples in which there is no target signal or a significantly reduced control product are diluted further until the level of inhibiting substances is suitably reduced.

In recent years we have applied our quantitative assays to ask a variety of questions about the colonization of *Verticillium* in various crops such as alfalfa or potato. Fig. 4 illustrates such a study in which the colonization of alfala by a non pathogenic potato isolate of *V. albo-atrum* was examined. In this case the results show that the fungus is confined largely to the stem base. Although a substantial amount of the fungus does survive in the lower stem for an extended period of time, a progressive decrease, however, indicates that mechanisms are present which lead to the eventual elimination of the fungus.

SOURCES OF CONTAMINATION AND FALSE POSITIVE SIGNALS

The very high sensitivity of PCR amplification raises special problems with false positive signals which usually do not occur with traditional methods. These are normally related to the stringency of the amplification conditions, the quality of the PCR reagents and the care with which assays are carried out. The first of these concerns can be addressed by a proper selection of target sequence and reaction protocol, particularly the annealing temperature. As noted earlier, the initial target sequence should be selected with care to ensure that it represents the pathogen of interest. Although the PCR-amplified DNA product can be detected by various colorimetric methods, product analysis by gel fractionation is highly recommended, with the product size offering an extra measure of certainty. The annealing temperature should be as high as possible to reduce the chance of less complementary hybrids while maintaining a reasonably efficient amplification level. This is especially critical when closely related species such as *V. albo-atrum* vs *V. dahliae* must be differentiated. For some primers, the amplification efficiency may have to be compromised by a higher annealing temperature to ensure a stringent reaction.

The quality of reagents and assay conditions including containment measures, have been reviewed widely (e.g., Kitchin and Bootman, 1993; Victor *et al.*, 1993) and are always of major concern. In the course of our studies we have made two very relevant observations repeatedly. DNAs from organisms being grown or cloned in the lab always are very widely distributed in the lab, including in the water supply. Similarly, if the lab is located in a farming community the local pathogens also are distributed widely in the lab, including the water supply. For example, on our campus which is located close to farming land, *V. dahliae* is found in the deionized water supplies of all the buildings, while water from the University of Toronto, in the center of a major city, does not show this contamination. As a result commonly we have linked contamination problems to water used in the preparation reagents and have been able to avoid these difficulties by taking water directly from a glass still using glass vessels which are baked at high temperatures.

Another especially critical point in our experience has been the micropipetters which are used to dispense reagents. In this case we have isolated sets of pipettes for reagent use only and never use them to pipette DNA samples. Alternatively, the use of pipettes with built-in filters is recommended although the cost is increased substantially.

The two specific concerns which are addressed have been most critical for us. However, because there is great controversy about containment methods for PCR assays, we highly recommend that researchers undertaking such assays review the literature for a range of opinions and ideas. Whatever precautions are adopted, the last and most recommended precaution is the constant use of both positive and negative controls. Reaction mixtures with and without internal control templates should always be included to ensure an efficient and contamination free assay.

CONCLUSIONS

We believe our success with a PCR-based assay for *Verticillium* in plant tissue and soil samples represents a good model for other developments of this type. The universal presence of high copy ribosomal RNA genes in all organisms makes these genes especially attractive targets for gene isolation and PCR amplification. A sample dilution strategy permits the direct extraction of DNA from either soil or tissue samples without further purification, a feature which permits low cost and wide scale applications. The use of internal control templates largely eliminates the possibility of false negatives and permits quantitative analysis, something which is usually very inaccurate or impossible with traditional assays. Finally the careful preparation of PCR reagents and the use of negative controls eliminate the possibility of false positives, a careful selection of primer sequence and annealling conditions permits the differentiation of even closely related species. With an aim at rapid, low cost, wide scale testing a number of compromises have been included in the approaches with satisfactory results. Recent applications to nematode diagnostics appear to support the general application of our approaches to many important crop pathogens.

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